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CELLULAR DEFENSE REACTIONS OF *PHASCOLOSOMA AGASSIZII*, A SIPUNCULAN WORM: PHAGOCYTOSIS BY GRANULOCYTES

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Abstract

Based on Wright's blood stain, uninucleate circulating granulocytes of the sipunculan *Phascolosoma agassizii* Keferstein are of two types: acidophils and basophils. The granulocytes are characterized by numerous granules scattered throughout the cytoplasm. Morphology of the granulocytes viewed with the light and electron microscope is illustrated and described. The granules of the granulocytes contain the enzymes acid phosphatase, alkaline phosphatase, lipase, and peroxidase. Fibrin-like strands entrap foreign particles—polystyrene latex beads and *Staphylococcus aureus*—introduced into the coelomic fluid *in vivo* or *in vitro*. Granulocytes accumulate in the vicinity of the trapped foreign particles. Both acidophilic and basophilic granulocytes phagocytize polystyrene latex beads and *Staphylococcus aureus in vivo* and *in vitro*. The cytoplasmic granules of the granulocytes degranulate into the phagocytic vacuoles and new granules form from the concave face of the Golgi complex.

Introduction

The phylum Sipuncula contains unsegmented marine worm-like animals that have a muscular body enclosing a coelomic cavity. The cavity contains the internal organs and a variety of circulating cell types: (1) hemocytes, nucleated cells containing the respiratory pigment hemerythrin, (2) uninucleate granulocytes, also called amoebocytes or leukocytes, (3) cell pairs—"cell within a cell" structures also containing granules (Dybas, 1975), (4) enigmatic vesicles—very large multinucleate or multicellular masses often surrounding a vesicle (Hyman, 1959), (5) ciliated urns, (6) immature cells that appear to be differentiating into other cell types, and (7) ova or spermatids (depending on the animal's sex) shed from the gonads into the coelomic cavity, where they develop. Some species lack one or more of these cell types.

Sipunculans are hardy animals capable of withstanding laboratory conditions and repeated injections and bleedings. Therefore, they have been used as experimental animals to investigate cellular and humoral immune reactions. In *in vivo* and *in vitro* studies, the granulocytes have been observed to be the main phagocytic cells in *Sipunculus nudus* (Cuénot, 1900; Cantacuzène, 1922; Volkonsky, 1933; Valembois and Boiledieu, 1980), *Phascolosoma vulgare* (Volkonsky, 1933), and *Phascolosoma agassizii* (Towle, 1962; Blitz, 1965). However, Brown and Winterbottom (1969) injected thorium dioxide into the coelomic cavity of *Golfingia capensis*, a species without ciliated urns, and observed that all coelomic cells ingested the thorium particles. Triplett *et al.* (1958) found that both autograph and homograph tentacle transplants into the coelomic cavity of *Dendrostomum zostericolum* became encapsulated. However, neither phagocytosis nor encapsulation was

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enhanced when second transplants were made. They concluded, therefore, that *D. zostericolum* lacked the capacity to synthesize antibody to a specific antigen. In another study (Cushing and Boraker, 1975), coelomocytes from *D. zostericolum* were capable of distinguishing "self" from "not self." Homologous eggs treated by heating, staining, or sonification were recognized as "not self" and encapsulated. Male sipunculan coelomocytes did not encapsulate untreated homologous eggs injected into their coelomic cavity; however, eggs possessed some antigens which also occurred on sperm.

Other coelomocyte types have also been associated with specific immunological reactions. In *S. nudus*, the ciliated urns secrete mucus that agglutinates and traps the foreign particles (Cantacuzène, 1922; Bang and Bang, 1962, 1965, 1974, 1975, 1976). Agranular or hyaline leukocytes of *S. nudus* have a cytotoxic effect against xenogenic hemocytes. Lysis requires close contact between the cells (Boiledieu and Valembois, 1977; Valembois and Boiledieu, 1980). Hemocytes of *D. zostericolum* have antigens capable of reacting with various human antisera (Cushing *et al.*, 1963).

Strong anti-bacterial properties have been demonstrated in the coelomic fluid of *S. nudus* (Bang and Bang, 1962), *Phascolosoma gouldii* (Bang and Krassner, 1958; Krassner, 1963), *Golfingia gouldii* (Rabin and Bang, 1964; Krassner and Florey, 1970), *P. agassizii* (Blitz, 1965), *D. zostericolum* (Johnson and Chapman, 1970), and *Dendrostomum pyroides* (Krassner and Florey, 1970). Injecting bacteria in *D. zostericolum* resulted in production of a non-specific bactericidin not present, or present in very low amounts, in non-injected animals (Evans *et al.*, 1969; Evans *et al.*, 1973). Injecting ciliates or bacteria caused rapid production of a lysin for some bacteria, ciliates, and blood cells in *S. nudus* (Cantacuzène, 1922; F. B. Bang, 1966, 1967), and *P. agassizii* (Blitz, 1965). Although *P. agassizii* contained a lysin for some gram positive and gram negative bacteria, it does not contain the lysin for the ciliate protozoan that Bang and Bang (1962) observed in *S. nudus* (Blitz, 1965). Cushing *et al.* (1969) showed that a substance they called stop factor (SF) in the coelomic fluid of *D. zostericolum* could immobilize a marine dinoflagellate within 10 min; recovery took 12 h. Weinheimer *et al.* (1970), demonstrated a naturally occurring lysin and hemagglutin for foreign red blood cells in the coelomic fluid of *D. zostericolum*. Foreign particles were also agglutinated in the coelomic fluid of *S. nudus* (Cantacuzène, 1922) and *P. agassizii* (Towle, 1962; Blitz, 1965). The bactericidins, lysins, and agglutinins can be separated from one another on the basis of characteristic properties, such as thermostability, enzyme sensitivity, and filtration.

The studies cited above show that sipunculans can launch both cellular (phagocytosis and encapsulation) and humoral (agglutinins, lysin, and bactericidin production) defense reactions. With the exception of the ciliated urn, which has been studied in some detail in *Phascolosoma scolops* (Ohuye *et al.*, 1961), *S. nudus* (Bang and Bang, 1962, 1965, 1974, 1975, 1976; Nicosia, 1979), and *P. agassizii* (Dybas, 1976), little is known about the specific functional roles or detailed morphology of the defense system. This study was undertaken to elucidate the role of the circulating granulocytes in the cellular immune response of *Phascolosoma agassizii*.

Materials and Methods

The 30 specimens of *Phascolosoma agassizii* Keferstein (Fig. 1) used in this study were collected from crevices in rocks exposed at low tide along Arroyo de

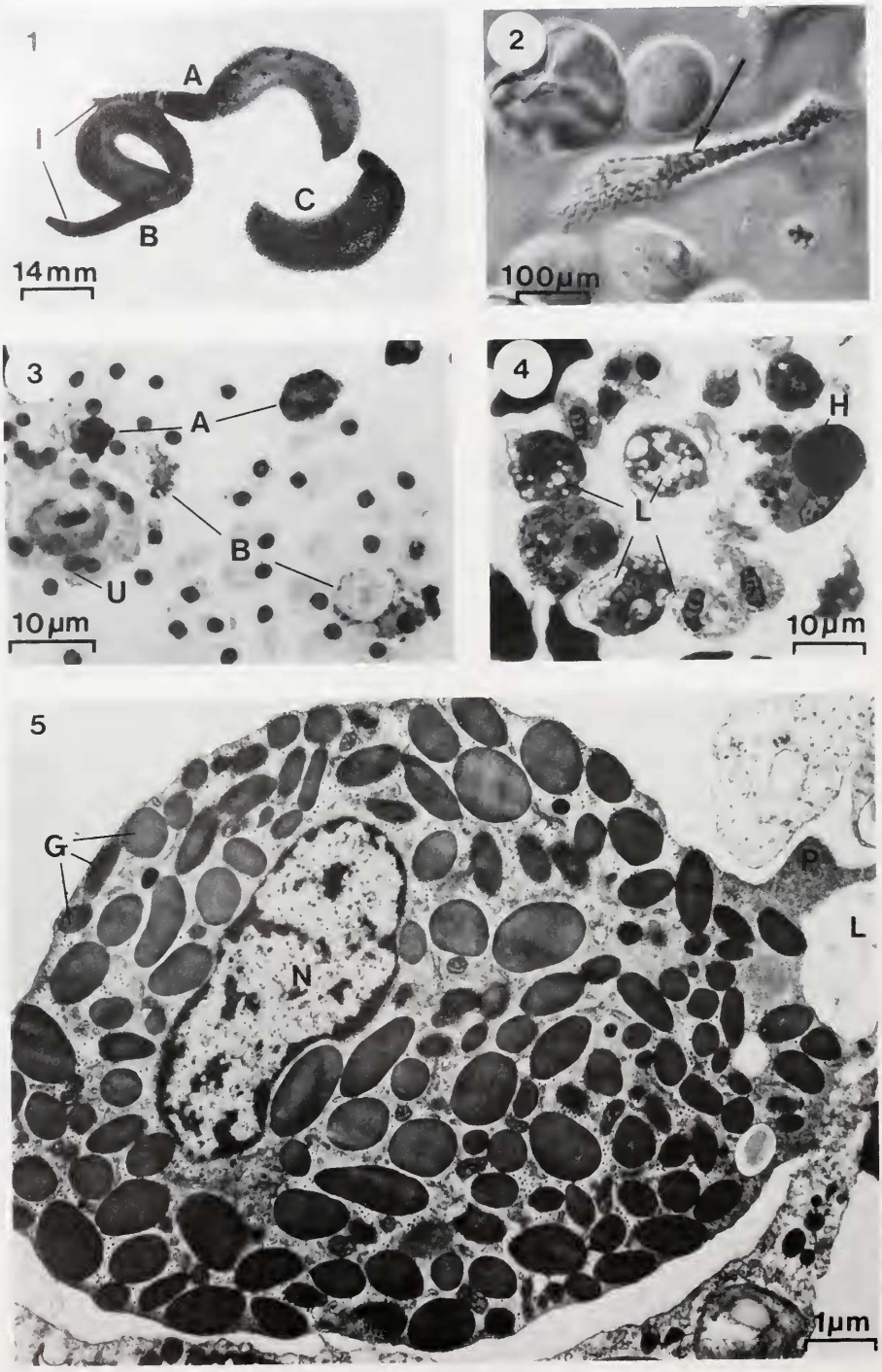

FIGURE 1. Whole specimens of *Phascolosoma agassizii*, A and B with introverts (I) extended, C with introvert retracted, exhibiting the characteristic "peanut" shape.

Frijoles State Beach, California. By placing the sipunculans on a dental wax sheet and cutting off the posterior 2–3 mm with an alcohol-cleaned razor blade, a large drop of coelomic fluid was obtained. This was pipetted into various solutions or quickly immersed in fixative.

Whole-cell preparations for light microscopy were made using a Shandon cytocentrifuge (Shandon Instr. Co., Sewickley, Penn.) designed for centrifuging cell suspensions directly on the slide. Cell suspensions were made from pooled samples of coelomic fluid (10 animals/sample). All tests were performed in triplicate. The slides were either air-dried and stained with Wright's blood stain (Lillie, 1969) or reacted for one of four different enzymes: (1) Goodpasture's reaction for peroxidase, omitting the fixative (1919), (2) Barka and Anderson's (1962) reaction for acid phosphatase, extending the incubation times from 45 min to 2–4 h, (3) Burstone's (1958) reaction for alkaline phosphatase, extending the incubation time to 4 h, or (4) Gomori's reaction for lipase from Yam *et al.* (1971). Material known to give a positive reaction for each enzyme (human bone marrow or rat kidney) was tested simultaneously with each sipunculan test sample. In other controls, the substrate or capture agent was omitted, or an inhibitor was added to the incubation medium: 0.01 *M* NaF for acid phosphatase, 0.001 *M* cysteine for alkaline phosphatase, and 0.01 *M* KCN for peroxidase (Bainton and Farquhar, 1968). After the enzyme reactions were completed, the slides were counterstained to enhance coelomocyte recognition (except for the acid phosphatase preparations, in which the reaction product was scant and faint), coverslipped, and examined under the light microscope. For a reaction to be classified as positive for an enzyme, the positive controls also had to be positive and the negative controls—both omission of substrate and addition of inhibitor—had to be negative.

For electron microscopy and light microscopy of Araldite-embedded coelomocytes, cells were fixed in 3% distilled glutaraldehyde buffered with 0.2 *M* sodium cacodylate in millipore-filtered seawater. After overnight fixation, cells were postfixed in 1% Michaelis-buffered osmium tetroxide (Caufield, 1957) for 90 min, dehydrated in graded ethanols, and embedded in Araldite (Luft, 1961). To facilitate handling, cells were packed by centrifuging (about 10,000 × *g*) with a Microfuge 152 (Beckman Instr., Inc., Spinco Div., Palo Alto, Calif.) (Malamed, 1963). Polymerized blocks were sectioned on a Sorvall Porter-Blum MT-2 Ultramicrotome (Dupont Corp., Norwalk, Conn.) equipped with glass knives.

For light microscopy, 0.5 μm sections were stained with 0.5% toluidine blue in sodium borate (Trump *et al.*, 1951). For electron microscopy, sections were placed on Parlodion and carbon coated grids (Hayat, 1970), double stained with 1% alcoholic uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963), and viewed with a Siemans Elmiskop IA. Electron micrographs were taken at original magnifications of 3000 to 30,000 times on Kodalith 70 mm Estar base film (Eastman Kodak Co., Rochester, N. Y.).

FIGURE 2. Phase contrast micrograph of an unfixed amoeboid granulocyte (arrow) showing the nucleus and cytoplasmic granules.

FIGURE 3. Whole air-dried coelomocytes stained with Wright's blood stain: acidophilic granulocytes (A), basophilic granulocytes (B), and a ciliated urn (U). The other cells in the field of view are hemocytes.

FIGURE 4. Light micrograph of several granulocytes that have phagocytized latex beads (L) and one that has phagocytized a hemocyte (H). Thick Araldite section stained with toluidine blue.

FIGURE 5. Fine structure of a typical granulocyte: nucleus (N), granules (G), pseudopodia (P) partially surrounding latex beads (L). Glycogen, rough endoplasmic reticulum, and mitochondria are dispersed throughout the cytoplasm.

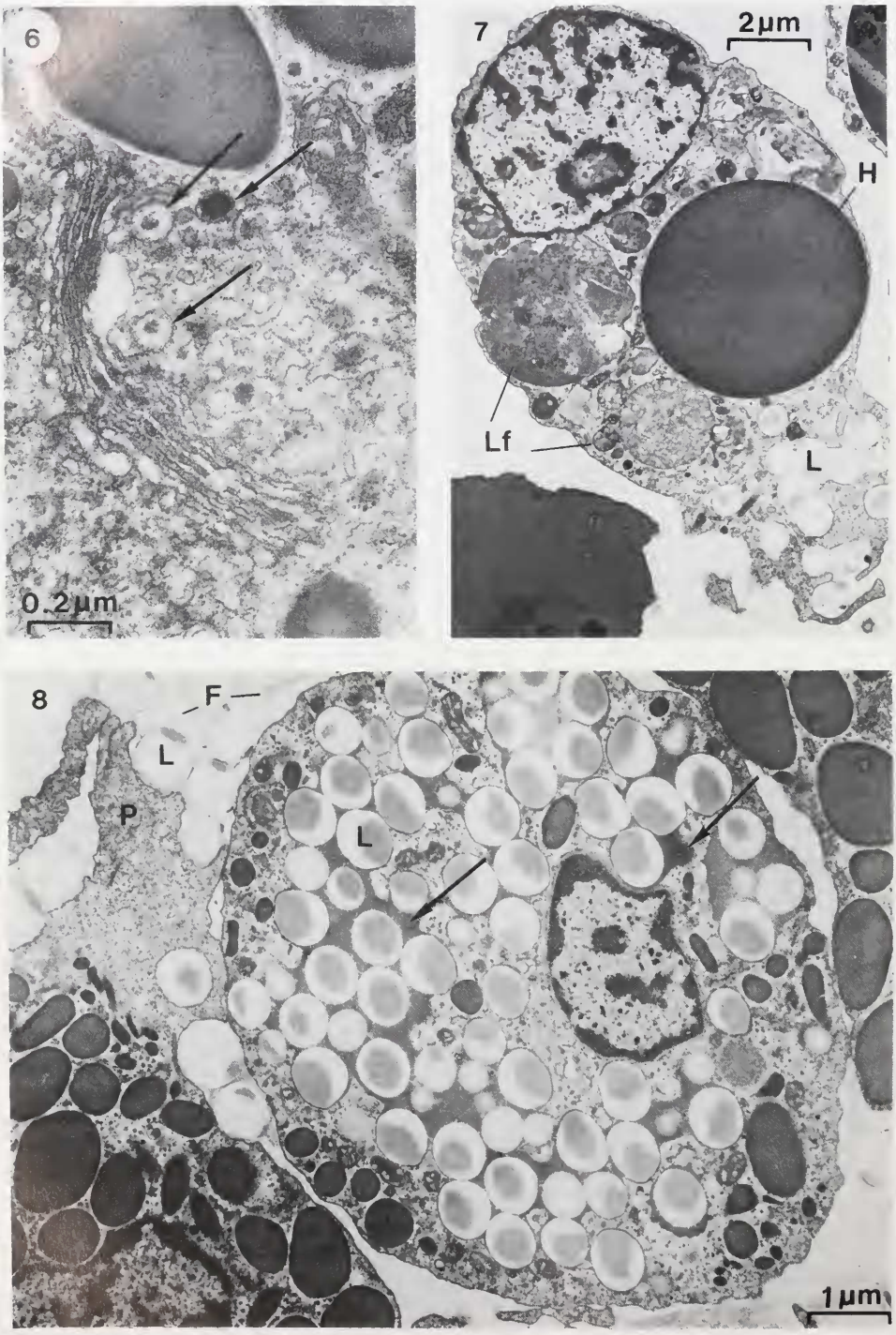

FIGURE 6. High magnification electron micrograph of a granulocyte 60 min after *in vitro* incubation with latex beads. New granules (arrows) are forming off the concave face of the Golgi complex.

For determining phagocytic cells, both *in vivo* and *in vitro* experiments were performed using polystyrene latex beads, size 0.79 μm, and heat-killed *Staphylococcus aureus* 502A, size 1.0 μm. In *in vivo* experiments, particles suspended in millipore-filtered seawater were injected in amounts of 0.1 cc through the body wall into the coelomic cavity. In *in vitro* experiments, the particles suspended in seawater were added to a test tube containing whole coelomic fluid at a concentration of approximately 10,000 bacteria or latex beads per milliliter of coelomic fluid. At 7, 30, and 60 min, samples of whole coelomic fluid were processed as previously described for electron microscopy. Other samples were viewed as living cells with the light microscope.

Results

In unfixed living cells (Fig. 2), some granulocytes (10.95 μm in diameter) ranged from colorless to pale green, and round to amoeboid in shape. Others, slightly larger (15.33 μm in diameter), contained granules varying from colorless to dark gold or orange.

In Wright's blood stain (Fig. 3), the granulocytes had either basophilic or acidophilic granules, but no single cell showed both staining reactions simultaneously. The size and number of granules varied. The cytoplasm of some cells was completely filled with granules, whereas in others granules were dispersed through the cytoplasm. In general, the smaller granulocytes contained numerous acidophilic granules, while the larger granulocytes contained basophilic granules. The basophilic granules varied more in size and in number than did the acidophilic granules.

In toluidine-blue stained sections (Fig. 4), the granules in the granulocytes all stained a uniform blue, regardless of size. Pseudopodia of amoeboid granulocytes were free of granules. The cytoplasm often contained phagocytized latex beads or *S. aureus* and what appeared to be phagocytized hemocytes.

In enzyme studies reactions were positive for acid phosphatase, alkaline phosphatase, lipase, and peroxidase. The positive reaction product was localized in the granules of the granulocytes. Attempts to stain for more than one enzyme on a slide were not successful.

The electron-dense granules in the granulocytes were the most distinguishing feature of the cells revealed by electron microscopy (Figs. 5–7). The granules all displayed a uniform density except in the immediate vicinity of a Golgi complex, where they showed a clear zone between the limiting membrane of the granule and its electron-dense core (Fig. 6). A nucleolus was occasionally encountered. Ribosomes were found along the perinuclear membrane, and rough and smooth endoplasmic reticulum was dispersed throughout the cytoplasm. Mitochondria, glycogen, and lipid droplets were also present. In many cells, residual bodies, lipofuscin-like pigments, and membrane whorls could be seen in the cytoplasm. Cell organelles or other cells were occasionally recognizable within a phagocytic vacuole (Fig. 7).

FIGURE 7. Fine structure of a granulocyte with varied cytoplasmic inclusions: lipofuscin-like material (Lf) which is thought to be insoluble residues of phagocytosis, a phagocytized hemocyte (H), and latex beads (L).

FIGURE 8. Electron micrograph of granulocytes 60 min after *in vitro* incubation with latex beads. In this plane of section, 74 latex beads (L) are visible within one cell, many within a single phagocytic vacuole. Most granules have already degranulated into the phagosome. The contents of the degranulated granules appears as dark material in the phagosome (arrows). Extracellular latex beads are trapped in a fibrin-like clot (F). Granule-free pseudopodia (P) from the cell left of center are beginning to surround the extracellular latex beads.

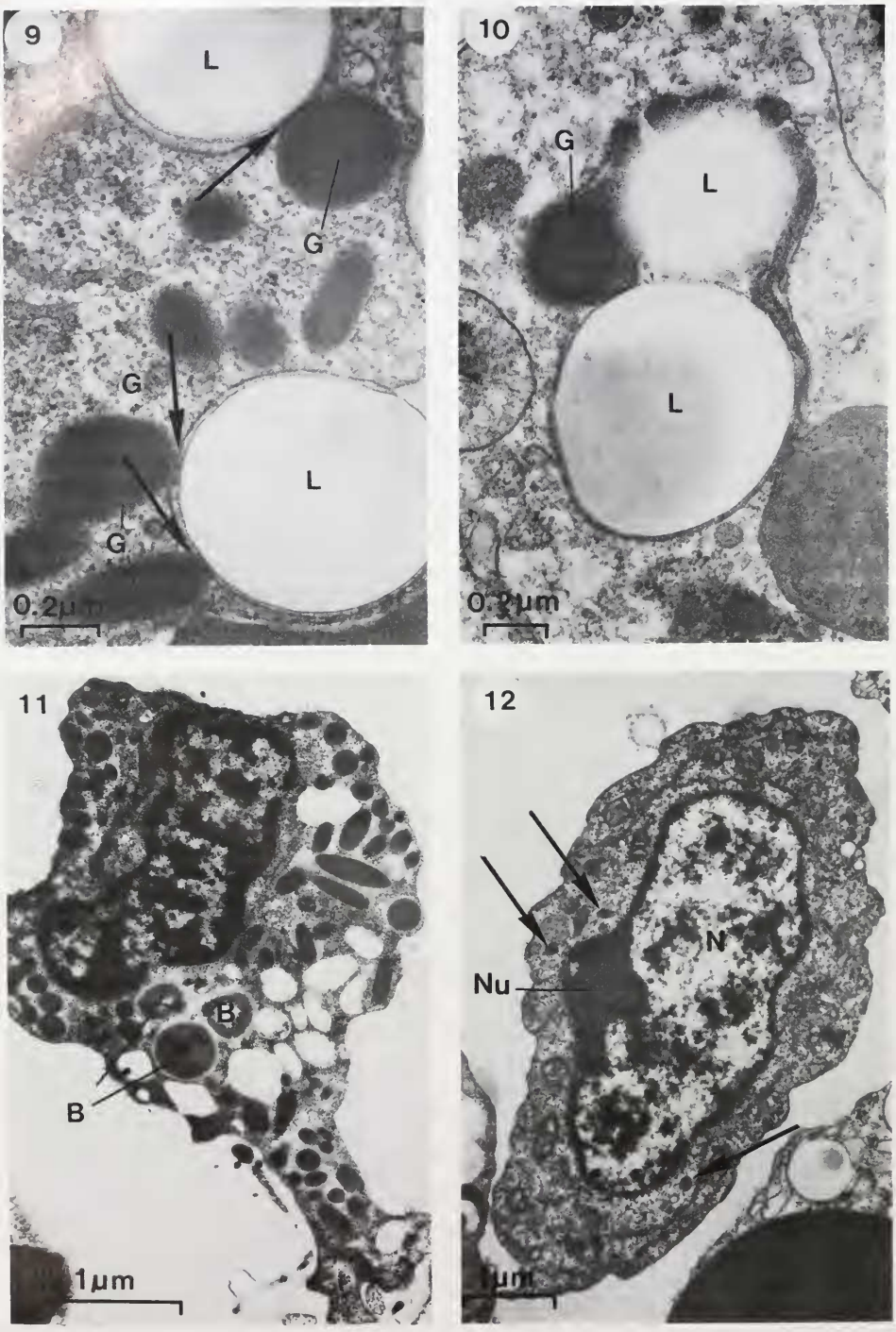

FIGURE 9. High magnification electron micrograph of a granulocyte 7 min after *in vitro* incubation with latex beads. The limiting membranes surrounding the granules (G) have fused with the membranes surrounding the phagocytic vacuoles (arrows). Within the phagosomes are latex beads (L).

In phagocytosis experiments (Figs. 4, 5, and 7–11), the reactions of the granulocytes in *in vivo* or *in vitro* experiments did not differ drastically. Immediately after injection of polystyrene latex beads or *S. aureus*, clots of fibrin-like strands appeared around the injected particles, trapping them and preventing their rapid dispersal. By 7 min, granulocytes were near the clotted foreign particles and pseudopodia were surrounding a latex bead (Figs. 4, 5, 7, and 8) or bacterium. Granulocytes were the dominant cell type surrounding the clotted foreign particles (Fig. 4). Most granulocytes already had foreign particles within their phagocytic vacuoles. By 30 min, extensive degranulation into the phagocytic vacuoles was apparent. Granules surrounded the phagosomes, and membranes surrounding the granules fused with membranes of the phagosome (Fig. 9). The granule contents that empty into the phagosome were visible at first as discrete condensed spheres adjacent to the inner side of the vacuole membrane (Fig. 10). Later, the contents dispersed throughout the phagocytic vacuole (Fig. 8). There were often numerous latex beads within a phagocytic vacuole.

By contrast, even after 60 min, although numerous *S. aureus* specimens had been phagocytized, it was rare to find even two of them in a phagocytic vacuole (Fig. 11). By 60 min, many granulocytes were almost completely degranulated. Golgi complexes were prominent and new granules appeared to be forming from the concave face of the Golgi complex (Fig. 6). Occasionally, granulocytes degranulated outside cells, into a clot of foreign particles, usually into clots that were too large to ingest. Although some granulocytes contained no phagocytized particles, all mature granulocyte types, regardless of size, number of granules, or other large inclusions, were observed with phagocytized particles. Blast-like cells (Fig. 12) that contained a few dense granules, suggesting that they were an immature form of granulocytes, were never observed containing recognizable phagocytized particles.

Discussion

Coelomic cell types vary somewhat within the phylum Sipuncula, but granulocytes have been reported in all species in which the coelomic fluid has been studied. In *P. agassizii* all uninucleate granulocytes, regardless of variation in number and size of granules, can phagocytize polystyrene latex beads or *S. aureus in vivo* and *in vitro*. In *Sipunculus nudus*, where the ciliated urn is a mucus-secreting cell thought to be the primary agglutinator in trapping foreign particles, granulocytes (amoebocytes) surround the mucus tails of the urns, acting as scavengers phagocytizing the entrapped particles (Bang and Bang, 1965; 1971). In *P. agassizii*, after 30 min incubation with the foreign particles, urns and granulocytes often surrounded clotted foreign particles, but granulocytes were the dominant cell type. This was also observed by Blitz (1965).

In the present study, granulocytes seemed attached to the clotted material. They remained surrounding the clot throughout the manipulations required for processing

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FIGURE 10. After degranulation, granule contents (G) appear inside the phagosome adjacent to the latex beads (L).

FIGURE 11. Fine structure of a granulocyte 30 min after in vitro incubation with heat-killed *Staphylococcus aureus*. Three bacteria (B) have been phagocytized.

FIGURE 12. Electron micrograph of a blast-like cell, possibly an immature form of a granulocyte. The nucleus (N) contains a prominent nucleolus (Nu). A few granules (arrows) are scattered throughout the cytoplasm.

cells for electron microscopy. Foreign particles were also trapped in the current created by the beating cilia of the urns and phagocytized by the cupola cells of the ciliated urn (Dybas, 1976).

It is not known whether the fibrin-like strands which trap foreign particles in *F. agassizii* originate from components of the coelomic fluid, or if the presence of one of the numerous cell types is required. Regardless of the fibrin's origin, clotting, which prevents dissemination of particles throughout the entire coelomic cavity, would have survival advantage if the foreign particles were pathogens.

The phagocytic cells reacted positively for enzymes that in vertebrates are involved in digestion (acid phosphatase, alkaline phosphatase, and lipase) or in killing microbes (peroxidation of bacterial cell walls) (Klebanoff, 1967; Weiss, 1977). These enzymes may serve the same function in sipunculan granulocytes. The considerable diversity of granule size within a granulocyte could represent either different populations of granules or different stages in packaging and development.

Since Golgi complexes were prominent in degranulated granulocytes, and only rarely seen in non-degranulated granulocytes, degranulation may trigger the formation of new granules.

With the electron microscope, granulocytes were often seen to contain residual bodies or large inclusions of undigested material. The fate of these inclusions is not known. However, Towle (1962) observed in a phagocytosis experiment that carmine dye was excreted via the nephridia. He concluded that eventually exocytosis rids a cell of indigestable material. Cushing (1963—personal communication cited in Blitz, 1965) and Blitz (1965) also observed that foreign material was excreted via the nephridia.

I have no data on the granulocytes' life spans. Even after repeated bleedings, no granulocytes or other cell types were seen in mitosis. Blast-like cells that did not seem to belong to any recognizable cell type had some characteristics suggesting that they may represent an ontogenetic stage of the granulocytes. These cells were small (6 μm in diameter). They had a large nucleolus adjacent to the nuclear membrane; a basophilic cytoplasm containing mostly free ribosomes, but also some rough endoplasmic reticulum; mitochondria; and a few widely scattered granules. They never contained recognizable phagocytozed particles.

The sipunculan granulocytes resemble vertebrate neutrophils in structure and function, although no phylogenetic relationship between sipunculan and vertebrate immune responses has been shown. Nevertheless, to survive, sipunculans must have developed an efficient mechanism to recognize foreign substances, rid the open coelomic cavity of possible pathogens, and recycle nutrients from cells that are no longer functional.

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